J. Phytopathology 145, 267–270 (1997)

ISSN 0931-1785

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Rapid and Sensitive Detection of *Xanthomonas fragariae* by Simple Alkaline DNA Extraction and the Polymerase Chain Reaction

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With 4 figures

Received October 9, 1996; accepted February 27, 1997

Abstract

Methods for DNA preparation from *Xanthomonas fragariae* in infected or artificially contaminated strawberry plants were compared in diagnostic assays using the polymerase chain reaction (PCR). The bacterium was detected using PCR with primers specific to a region of its *hrp* gene. Sensitivity of detection was 1.25×10^3 CFU ml^{-1} using DNA from bacterial suspensions prepared by an alkali extraction method. This was 10-fold more sensitive than DNA extraction by boiling, and was equal to that in which DNA was prepared by a more involved cetyltrimethylammonium bromide (CTAB) procedure. Sensitivity of detection from artificially contaminated strawberry tissues was 10-fold less than that from cell suspensions. The results indicated that a rapid and simple method of alkali DNA sample preparation is applicable for the sensitive and reliable detection of *X. fragariae* and possibly other plant pathogenic bacteria.

Zusammenfassung

Eine schnelle und empfindliche Nachweismethode für *Xanthhomonas fragariae* durch eine einfache alkalische DNA-Extraktion und die Polymerase-Kettenreaktion

Mit Hife der Polymerase-Kettenreaktion (PCR) wurden Methoden verglichen, die die DNA von *Xanthomonas fragariae* aus infizierten bzw. künstlich kontaminierten Erdbeerpflanzen extrahieren sollen. Das Bakterium wurde durch PCR mit spezifischen Primern für einen Teil dessen *hrp*-Gens nachgewiesen. Die Nachweisempfindlichkeit lag bei $1{,}25 \times 10^3$ cfu (koloniebildende Einheiten)/ml bei der Verwendung der DNA aus bakteriellen Suspensionen, präpariert mit Hilfe einer alkalischen Extraktionsmethode. Diese Methode war 10-fach empfindlicher als eine DNA-Extraktion durch Kochen und vergleichbar mit der aufwendigenre Cetyltrimethylammoniumbromid (CTAB)-Methode. Die Nachweisempfindlichkeit bei künstlich kontaminiertem Erdbeergewebe war 10-fach niedringer als der Nachweis aus Zellsuspensionen. Die Ergebnisse deuten darauhin, daß eine schnelle und einfache alkalische Methode der DNA-Vorbereitung für den empfindlichen und zuverlässigen Nachweis von *X. fragariae* und möglicherweise anderen bakteriellen Pflanzenkrankheitserregern geeignet ist.

Introduction

Xanthomonas fragariae, a member of the *Pseudomonadaceae*, is a phytopathogenic bacterium which causes angular leafspot disease of Fragaria plants (Hazel et al., 1980). Cultivated strawberry, *Fragaria* × *ananassa*, is its most important host.

Since angular leafspot disease of strawberry was first described (Kennedy and King, 1960), its occurrence and geographical distribution has increased (Rowhani et al., 1994). All cultivars of strawberry tested so far are susceptible, and infection of all plant parts except roots has been observed (Hazel et al., 1980). The pathogen appears to be disseminated in contaminated planting stock (Maas, 1984). Therefore, proper inspection of strawberry plants is required to satisfy quarantine restrictions.

Typical symptoms of angular leafspot are initially watersoaked angular spots on lower leaf surfaces which eventually occur on the upper leaf surface as irregular reddish-brown spots followed by necrosis. Under certain conditions, such as at high elevations, crown rot and vascular collapse (systemic infection) may occur (Hildebrand et al., 1967). At this stage, watersoaking appears at the base of newly emerging leaves before sudden death of the plant. Symptoms at the foliar phase of the disease are difficult to distinguish from those of common leafspot (caused by *Mycosphaerella fragariae*) and leaf scorch (caused by *Diplocarpon earliana*), whereas a vascular collapse can also be caused by *Phytophthora cactorum*. Thus, visual inspection of strawberry plants is insufficient for sanitary control.

In addition to visual inspection, the bacterium is com-

monly isolated and tested for pathogenicity. However, this is difficult because the pathogen grows very slowly and is easily overgrown by saprophytes, and pathogenicity tests are time-consuming. Enzyme-linked immunosorbent assay (ELISA) is a rapid alternative but is also less sensitive than the pathogenicity test (Rowhani et al., 1994).

The polymerase chain reaction (PCR) is now an important technique for the detection of a range of pathogens in their plant hosts (e.g. Henson and French, 1993; Audy et al., 1996). A PCR assay developed for *X. fragariae* is rapid and less time-consuming than traditional assays, and appears to be more sensitive than ELISA (Roberts et al., 1995).

For inspection purposes, fast and simple sample preparation that does not require highly toxic compounds is important. DNA sample preparation by alkali treatment was developed initially for monitoring transgenic sequences in plants by PCR (Klimyuk et al., 1993). A similar method was recently reported for the detection of *Xanthomonas campestris* pv. *phaseoli* in bean seeds (Audy et al., 1996). However, little information on the relative sensitivity of the method was reported. Here we report a simple and rapid method of DNA sample preparation for the sensitive and reliable detection of *X. fragariae* using PCR.

Materials and Methods

Growth and maintenance of bacterial cultures

X. fragariae 33239, originally from infected strawberries in California, was grown at 28°C on a medium containing 2.3% (w/v) nutrient agar (Difco, Toronto), 0.5% (w/v) glucose and 0.1% (w/v) yeast extract. A bacterial suspension was cultured in a 100 ml flask containing 20–50 ml medium of 1.0% yeast extract, 2.0% dextrose, and 2.0% $CaCO_3$ (YDC) (Stolp and Starr, 1964) on a rotary shaker at 100 r.p.m. at 30°C for 48 h.

Bacterial concentration

The bacteria were transferred from agar culture and resuspended in sterile phosphate-buffered saline (PBS: 8.0 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, and 0.2 g KCl per l, pH 7.2) containing 0.1% (v/v) Tween-20 (PBS-T). The initial concentration of the bacteria was adjusted to $A_{600} = 0.1$ to be 2.5×10^7 CFU ml^{-1} as determined by serial dilution and plating on YDC agar. Colony numbers were counted 6 days after plating, and bacterial concentrations were based on an average of at least three counts.

Pathogenicity tests

The bacterial suspension was diluted to 10^6 CFU ml^{-1} and inoculated onto the lower leaf surface of strawberry plants by spray infiltration. Two weeks after inoculation, the leaves with lesions were surface-sterilized with 0.5% sodium hypochlorite and ground in sterile PBS-T using a mortar and pestle. The homogenate was plated onto agar medium and incubated at 28°C.

Preparation of DNA samples for PCR

Three methods of DNA extraction were tested: the cetyltrimethylammonium bromide (CTAB) method described by Murray and Thompson (1980), the NaOH/HCl method and the boiling method. For the NaOH/HCl method, strawberry tissues (leaves, sepals, crowns) were excised and collected in Eppendorf tubes, containing 50 µl 0.25 N NaOH. The samples were boiled for 2 min and then 50 µl 0.25 N HCl was added, followed by 25 µl 0.5 M Tris-HCl (pH 8.0) plus 0.1% (v/v) Tween-20. After boiling for another 2 min and brief centrifugation, 1 µl of the supernatant and a 50-fold dilution of the supernatant in sterile distilled water was used in PCR. Serial dilutions of the bacterial suspension (100 µl) either alone or mixed with strawberry tissues were also treated as above. For the boiling method, dilutions of the bacterial suspensions (100 µl) or infected leaf tissue was mixed with 100 µl of the PBS-T solution and boiled for 10 min. After brief centrifugation, 1 µl of the supernatant or 50-fold dilution of the supernatant was used in PCR.

PCR analysis

Primers of JJ9 (5′-TGGGCCATGCCGGTGGAACT GTGTGG-3′) and JJ12 (5′-TCCCAGCAACCCAGA TCCG-3′) specific to a region of the *hrp* gene of *X. fragariae* were used for PCR amplification of *X. fragariae* DNA from infected strawberry tissues and bacterial suspension cultures. PCR was carried out with 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min. The reaction contained 200 µM of dNTPs, 1.0 pmoles/µl of each primer, 1.0 µl of the DNA sample, 0.7 unit/25 µl AmpliTaq (Perkin-Elmer Cetus, Mississauga, Ontario, Canada) in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin. Following agarose gel electrophoresis and ethidium bromide staining, PCR products were visualized under UV light (300 nm). Southern blot hybridization of the PCR products with ^{32}P-labelled PCR products amplified from the genomic DNA of *X. fragariae* 33239 was done as described in Sambrook et al. (1989).

Strawberry plants for PCR diagnosis

Strawberry plants without symptoms of angular leafspot disease were kindly provided by growers from various locations in Ontario and Nova Scotia, Canada and in Massachusetts, USA. They were briefly stored at 4°C before being assayed for the presence of *X. fragariae*. These samples were cv. Kent from Brantford, Ontario and Whately, Massachusetts, cvs. Annapolis and Mic Mac from Peterborough, Ontario, cv. Honeoye from Brampton, Nova Scotia and Thorndale, Ontario, cv. Annapolis from Woodstock, Ontario, cv. Cavendish from Waterford and Peterborough, Ontario, cv. Veestar from Thorndale, Ontario, and cv. Jewel from Whately, Massachusetts. Strawberry plants infected with *X. fragariae* 33239 showed symptoms of typical angular leafspot disease under glasshouse conditions.

Results

DNA sample preparation and PCR analysis

DNA was prepared from 10-fold serial dilutions of the bacterial suspension by the NaOH/HCl and boiling methods. Following PCR, a band of amplified DNA with

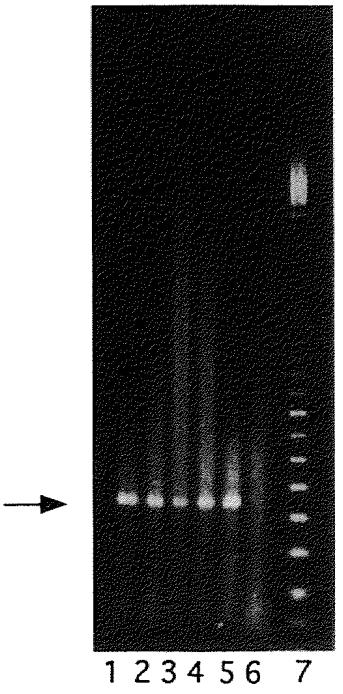

Fig. 1 Ethidium bromide stained agarose gel showing PCR amplification of *X. fragariae* DNA prepared from serial dilutions of the bacterial suspension by the NaOH/HCl method. The bacteria were serially diluted 10-fold from 1.25×10^7 CFU ml^{-1} in lane 1 to 1.25×10^2 CFU ml^{-1} in lane 6. Lane 7 shows DNA markers of 100 bp ladder. PCR-amplified DNA of 478 bp is indicated by an arrow

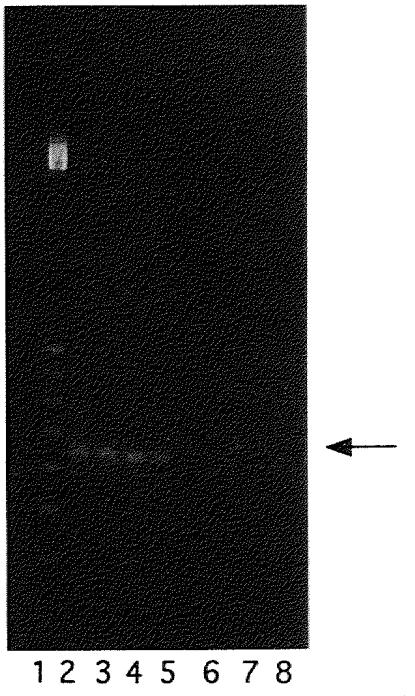

Fig. 2 Ethidium bromide stained agarose gel showing PCR amplification of *X. fragariae* DNA prepared from serial dilutions of the bacterial suspension by the boiling method. The bacteria were serially diluted 10-fold from 1.25×10^7 CFU ml^{-1} in lane 2 to 1.25×10^1 CFU ml^{-1} in lane 8. Lane 1 shows DNA markers of 100 bp ladder. PCR-amplified DNA of 478 bp is indicated by an arrow

the predicted size of 478 bp was produced by the DNA samples prepared by both methods. Further hybridization of the DNA with a ^{32}P-labelled DNA probe of the PCR product from *X. fragariae* genomic DNA also showed that the amplification was pathogen-specific. However, the NaOH/HCl method resulted in a detection sensitivity of 1.25×10^3 CFU ml^{-1} (Fig. 1), compared to only 1.25×10^4 CFU ml^{-1} by the boiling method (Fig. 2). The CTAB method was as sensitive as the NaOH/HCl method, but samples did not require dilution prior to PCR whereas those prepared by the other two methods needed to be diluted 50-fold to prevent PCR inhibition. Excised portions of leaves and crowns from symptomless strawberry plants were mixed with 100 μl of 10-fold serial dilutions of the bacterial suspensions and allowed to dry. DNA samples were prepared from these contaminated tissues by the NaOH/HCl method. PCR amplification of these DNA samples showed that the bacterium is detectable up to levels of 1.25×10^4 CFU ml^{-1} and 1.25×10^6 CFU ml^{-1} from contaminated leaves (Fig. 3) and crowns (Fig. 4), respectively.

PCR diagnosis of strawberry plants

Apparently healthy strawberry plants of several varieties were randomly collected from strawberry-growing areas in eastern Canada and USA. DNA samples from leaves, sepals and crowns prepared by the NaOH/HCl method were tested for the presence of *X. fragariae* by PCR amplification. All DNA samples from symptomless

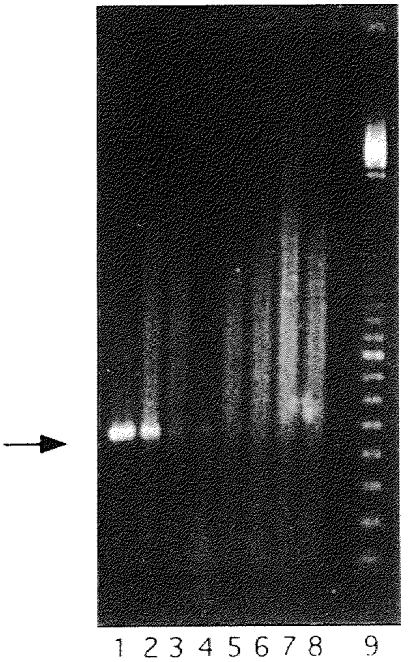

Fig. 3 PCR amplification of DNA from the leaf tissues contaminated with a 10-fold serial dilution of *X. fragariae* suspension. The bacterial concentrations of lanes 1–8 are 1.25×10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10 °CFU ml^{-1}. Lane 9 shows DNA markers of 100 bp ladder. PCR-amplified DNA of 478 bp is indicated by an arrow

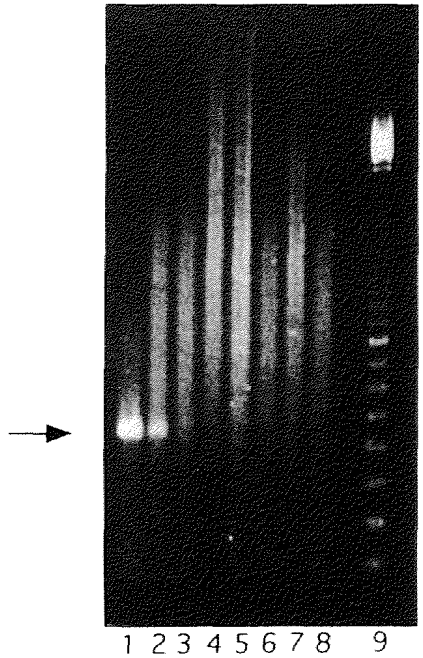

Fig. 4 PCR amplification of DNA from the crown tissues contaminated with a 10-fold serial dilution of *X. fragariae* suspension. The bacterial concentrations of lanes 1–8 are 1.25×10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10 °CFU ml^{-1}. Lane 9 shows DNA markers of 100 bp ladder. PCR-amplified DNA of 478 bp is indicated by an arrow

crowns, leaves and sepals were negative except those from leaves of cv. Veestar from Woodstock and cv. Canvendish from Waterford, and sepals of cv. Honeoye from Thorndale. PCR amplification of DNA from these tissues repeatedly gave a DNA product of the expected size.

Strawberry plants with leaf lesions typical of *X. fragariae* were also tested. These lesions were of different sizes, colouration and degree of watersoaking; each lesion was excised from the leaves for DNA preparation and subsequent PCR analysis. The bacterium was detectable by PCR from smaller lesions (1–3 mm in diameter) which were watersoaked and lightly coloured, but not from larger lesions (more than 5 mm in diameter) which were dry and darkened. This was highly reproducible with lesions taken from many different plants.

Discussion

Alkali treatment of strawberry tissues, followed by HCl neutralization (NaOH/HCl method), is a simple and rapid way to prepare DNA samples for detection of *X. fragariae* by PCR. Unlike the CTAB method, no expensive chemicals or organic solvents are required, and all stages of sample preparation are performed in a single tube. For large numbers of samples, the top of the microcentrifuge tube can be used quickly to excise a portion of the plant for subsequent PCR analysis or for storage at 4°C. Alkali treatment may be effective because it results in better denaturation of the DNA, so that the DNA template is more accessible to the Taq polymerase (Klimyuk et al., 1993). This method could possibly also be used for rapid PCR-based diagnosis of bacterial pathogens other than *X. fragariae*.

PCR has proved to be a reliable and sensitive method for detection of a range of pathogens (Henson and French, 1993). In this study, PCR was extended to the detection of *X. fragariae* from bacterial suspensions and strawberry tissues. The sensitivity of 1.25×10^3 CFU ml^{-1} by PCR is higher than that of 10^5 CFU ml^{-1} by ELISA (Rowhani et al., 1994).

The bacterium was also detected in symptomless plants by PCR. This indicates that symptomless *X. fragariae*-infected plants can be identified by PCR and shows the importance of using a sensitive assay to inspect symptomless strawberry plants for efficient control of the pathogen by preventing the spread of contaminated planting stocks (Calzolari and Mazzucchi, 1989).

Acknowledgements

The authors thank Pam Fisher, Bill Trerwith, Margaret Appleby, Tom Pate and Joe Uyenaka for supplying strawberry plants.

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